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REMARKS UNDER 37 CFR § 1.116

Formal Matters

Claims 17-19 are pending after entry of the amendments set forth herein.

Claims 17-22 and 26-29 were examined. Claims 17-22 and 26-29 were rejected. No claims were allowed.

Claims 1-16, and 20-44 have been canceled without prejudice.

Claim 17 has been amended. Support for the amendment can be found in the claims as originally filed and throughout the specification, at for example, page 56, line 8, through page 57, line 26.

The specification has been amended to address the objections raised by the Examiner. The specification has been amended on page 1, line 2 to reflect that U.S. Patent Application No. 09/525,965 has been abandoned. The specification has also been amended on page 8, line 3 to insert the sequence number.

Applicants respectfully request reconsideration of the application in view of the amendments and remarks made herein.

No new matter has been added.

Specification

The specification has been amended to address the objections raised by the Examiner. Withdrawal of these objections is respectfully requested.

Rejection under 35 U.S.C. § 112 first paragraph

Claims 17-21 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification allegedly failed to enable the claimed invention.

Without conceding as to the correctness of this ground of rejection, Applicants note that claims 20-21 have been cancelled, rendering the rejection moot as applied to these claims. Furthermore, applicants have amended Claim 17 for clarification. Claim 17 now requires that the viral vector is a recombinant adeno-associated virus. This rejection is traversed as it may be applied to the claims as presently pending.

The law is clear that "[t]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." United States v. Telectronics, Inc., 8 USPQ 2d 1217, 1233 (Fed. Cir. 1988), cert. denied, 490 U.S. 1046 (1989). See also, Genentech, Inc. v. Novo Nordisk, 42 USPQ 2d 1001 (Fed. Cir. 1997), cert. denied, 522 U.S. 963 (1997); Scripps Clinic and Research Foundation v. Genentech, Inc., 18 USPQ 2d 1001 (Fed. Cir. 1991). .

Furthermore, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. In re Certain Limited-Charge Cell Culture Microcarriers, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia, 227 USPQ 428 (Fed. Cir. 1985). See also, MPEP §2164.01. Practitioners in the chemical and molecular biology arts frequently engage in extensive modification of reaction conditions and complex and lengthy experimentation where many factors must be varied to succeed in performing an experiment or in producing a desired result. The Federal Circuit has found that such extensive experimentation is not undue in the molecular biology arts. For example, in Hybritech v. Monoclonal Antibodies, Inc. (231 USPQ 81 (Fed. Cir. 1986)) the court concluded that extensive screening experiments, while being voluminous, were not undue in view of the art which routinely performs such long experiments.

The Office Action asserts that the specification while being enabling for a method of inhibiting neovascularization in the eye, comprising the intraocular co-administration of an AAV-vector which directs the expression of anti-angiogenic factors (i.e. VEGF and Flt-1), does not reasonably provide enablement for a method of inhibiting neovascular disease of the eye comprising intraocular delivery of genes which direct the expression of an anti-angiogenic factor such that the disease is inhibited. These various aspects of the rejection are addressed below.

A. Inhibition of Neovascularization

In particular, the Office Action argues that because Applicants use a rat model which does not exhibit the disease, co-administration of anti-angiogenic factors cannot be shown to affirmatively rescue or ameliorate the disease. That is, according to the Office Action, the rat model used in the present invention is not an efficacious model of neovascular ocular disease because the animal does not develop the disease, and therefore amelioration of such disease cannot be proven.

Pending Claims 17-19 of the present invention are specifically directed to ***inhibiting neovascularization*** in the eye by production of an anti-angiogenic polypeptide in the eye, which production is accomplished by administering a recombinant adeno associated viral vector adapted for expression of the anti-angiogenic polypeptide in the eye of the subject. Applicants respectfully note that the present claims are directed to ***inhibition*** of ***neovascularization***. Other clinical signs or symptoms of the underlying disease may still be present, and the cause of the disease *per se* may still be present; however, neovascularization is inhibited in the subject having a disease.

Inhibiting neovascularization, which may or may not be accompanied by treating existing damage associated with neovascularization, is of great value. By analogy, administration of insulin to a diabetic subject can correct or help maintain glucose levels in the subject without curing the underlying cause of the disease. There is no question that there is value in treating or preventing abnormally high glucose levels in a diabetic subject – even though the disease of diabetes may still be present. In this case, thus, whether existing disease is treated or not, the ability to inhibit neovascularization so as to, at least, prevent further damage, is of great value.

As noted in the Office Action (page 4) the present application shows that subretinal injection of an AAV vector containing VEGF in a rat produced subretinal and choroidal neovascularization (Example 16). Moreover, the specification also shows that the co-injection of a vector including soluble Flt-1, and anti-angiogenic factor, with a vector including VEGF, an angiogenic factor, resulted in an inhibition of neovascularization (Example 17).

Accordingly, the evidence provided in the specification clearly establishes that (1) an anti-angiogenic factor inhibits neovascularization; (2) an angiogenic factor induces vascualrization; (3) when a anti-angiogenic factor is administered with a angiogenic factor, which angiogenic factor administered alone results in neovascularization, neovascularization is in fact inhibited. Therefore, it is reasonable to conclude that that administration of an anti-angiogenic factor inhibits neovascularization of the eye. Applicants respectfully submit that the claims are supported by an enabling disclosure in the specification for at least this reason.

Furthermore, research articles published prior to the filing of the present application support the Applicant's assertion that anti-angiogenic factors can be used to ***inhibit neovascularization*** in the eye. For example, Mori et al. (Exhibit A), showed that pigment epithelium-derived factor (PEDF) inhibits choroidal neovascularization when delivered using adeno associated viral vector. Specifically, Mori et al. showed that in mice which had received the anti-angiogenic factor PEDF, choroidal neovascularization was inhibited following insult to the retina in the form of laser induced choroidal neovascularization. In addition, Raisler et al. (Exhibit B), showed that Kringles 1-3 of angiostatin, also reduce retinal neovascularization when delivered using adeno associated viral vector. In particular, Raisler et al., showed that showed that in mice which had received the anti-angiogenic factor, choroidal neovascularization was reduced following insult to the retina in the form of elevated oxygen exposure

According, Applicants respectfully submit that, in providing evidence that choroidal neovascularization is inhibited by providing for expression of an anti-angiogenic factor in the eye, applicants have enabled the entire scope of the claim.

B. Anti-Angiogenic Factor

In addition, the Office Action asserts that the present application does not provide enablement for the use of any anti-angiogenic factor other than soluble flt-1.

The specification at, for example, pages 19-20 provides detailed information regarding a variety of other anti-angiogenic factors that are suitable for use with the claimed invention. In particular, the specification provides a list of well known and characterized anti-angiogenic

factors, as well as providing the citations fort research articles discussing the various anti-angiogenic factors.

Compliance with the enablement requirement under 35 U.S.C. §112, first paragraph does not require or mandate that a specific example be disclosed. The specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art would be able to practice the invention without undue experimentation.[1] Furthermore, "[n]othing more than objective enablement is required, and therefore it is irrelevant whether [a] teaching is provided through broad terminology or illustrative examples."[2]

Furthermore, research articles published subsequent to the filing of the present application support the Applicant's assertion that other anti-angiogenic factors can be used according to the full scope of the claims. For example, Mori et al. (Exhibit A), showed that pigment epithelium-derived factor (PEDF) inhibits choroidal neovascularization when delivered using adeno associated viral vector. In addition, Raisler et al. (Exhibit B), showed that Kringles 1-3 of angiostatin, also reduce retinal neovascularization when delivered using adeno associated viral vector.

Accordingly, based on the guidance provided in the specification of the present application and the relevant art, it would be reasonable to conclude that other anti-angiogenic factors, other than soluble flt-1, which was exemplified in Example 16, are suitable for use with the claimed invention. Therefore, the present specification provides an enabling disclosure to practice the claimed invention.

Therefore, the Applicants submit that the rejection of claims 17-21 under 35 U.S.C. §112, first paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

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1. *In re Borkowski,* 164 U.S.P.Q. 642, 645 (CCPA 1970).
2. *In re Robins* 166 U.S.P.Q. 552, 555 (CCPA 1970).

Rejection under 35 U.S.C. § 102

The Applicants acknowledge with gratitude the Examiner's indication that the rejection under 35 U.S.C. § 102 has been withdrawn.

Rejection under 35 U.S.C. § 103

Claims 22 and 27-28 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Kendall et al. in view of Bujard et al. Without conceding to the correctness of the rejection and in the spirit of expediting prosecution, claims 22, 27, and 28 have been canceled, rendering this rejection moot.

Conclusion

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number BERK-010CIP.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: June 4, 2004

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AAV-Mediated Gene Transfer of Pigment Epithelium-Derived Factor Inhibits Choroidal Neovascularization

Keisuke Mori,[1] Peter Gehlbach,[1] Satoru Yamamoto,[1] Elia Duh,[1] Donald J. Zack,[1,2] Qingbong Li,[3] Kenneth I. Berns,[3,4] Brian J. Raisler,[3] William W. Hauswirth,[3,4,5] and Peter A. Campochiaro[1]

Purpose. Adeno-associated viral (AAV) vectors have been used to express several different proteins in the eye. The purpose of this study was to determine whether AAV-mediated intraocular gene transfer of pigment epithelium-derived factor (*PEDF*) inhibits the development of choroidal neovascularization (CNV) in a murine model.

Methods. C57BL/6 mice were given intravitreous or subretinal injections of a PEDF expression construct packaged in an AAV vector (AAV-chicken β-actin promoter-exon 1-intron 1[CBA]-PEDF) or control vector (AAV-CBA-green fluorescent protein[GFP]). After 4 or 6 weeks, the Bruch's membrane was ruptured by laser photocoagulation at three sites in each eye. After 14 days, the area of CNV at each rupture site was measured by image analysis. Intraocular levels of PEDF were measured by enzyme-linked immunosorbent assay.

Results. Four to six weeks after intraocular injection of AAV-CBA-PEDF, levels of PEDF in whole-eye homogenates were 6 to 70 ng. The average area of CNV at sites of the Bruch's membrane rupture showed no significant difference in eyes injected with AAV-CBA-PEDF compared with uninjected eyes. In contrast, 4 to 6 weeks after intraocular injection of 1.5×10^9 or 2.0×10^{10} particles of AAV-CBA-PEDF, the area of CNV at the Bruch's membrane rupture sites had significantly decreased compared with CNV area at rupture sites in eyes injected with AAV-CBA-GFP.

Conclusions. These data suggest that intraocular expression of PEDF or other antiangiogenic proteins with AAV vectors may provide a new treatment approach for ocular neovascularization. (*Invest Ophthalmol Vis Sci.* 2002;43:1994–2000)

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Ocular neovascularization is a major threat to vision and a complicating feature of many eye diseases. In fact, choroidal neovascularization (CNV) complicating age-related macular degeneration (AMD) is the most common cause of severe visual loss in people older than 60 years in developed countries.[1] At best, current treatments merely delay severe vision loss, because they are directed at destroying new vessels and do not address the underlying angiogenic stimuli that frequently cause recurrences.

Currently, there are no antiangiogenic treatments available for patients with ocular neovascularization, but several new approaches hold promise. Orally active drugs that inhibit VEGF receptor kinases cause dramatic inhibition of ocular neovascularization in mice.[2–4] However, before this can be applied in patients, extensive safety data are needed to be certain there are no serious side effects from systemic inhibition of angiogenesis. To avoid these concerns, local delivery of several agents is being investigated. Phase I clinical trials testing the safety and tolerability of intraocular injections of an aptomer that binds VEGF or an anti-VEGF antibody have been completed, and phase II trials are being planned. Preliminary reports suggest that inflammation may occur, particularly after injection of the anti-VEGF antibody, but it is not considered a severe enough problem to discontinue these approaches.[5,6] Endogenous proteins are likely to be better tolerated, and, recently, several proteins with purported antiangiogenic activity have been identified,[7–12] and intraocular injection of each of these alone or in combination could be considered. However, the use of large molecules, such as aptomers or proteins, has a major disadvantage of requiring repeated intraocular injection.

Gene transfer offers an alternative means for local delivery of therapeutic proteins to intraocular tissues. Because the eye is a relatively isolated compartment, intraocular injection of a small fraction of the amount of viral vector used for systemic injections results in transduction of a large number of ocular cells and no transduction of cells outside the eye. Recently, we have demonstrated that intraocular injection of an expression construct for pigment epithelium-derived factor (PEDF) packaged in an adenoviral vector inhibits ocular neovascularization in three different mouse models.[13] This provides proof of concept for the gene transfer approach of treating ocular neovascularization, but adenoviral vectors have features that may limit their use in humans, including some evidence of toxicity and decreased transgene expression to low levels over the course of a few months. It is not yet known whether repeated intraocular injections of adenoviral vectors can be considered. Prolonged transgene expression with no evidence of toxicity has been demonstrated after intraocular injection of expression constructs packaged in adeno-associated viral (AAV) vectors.[14,15] In this study, we tested the effect of intraocular injection of AAV vectors containing expression constructs coding for PEDF.

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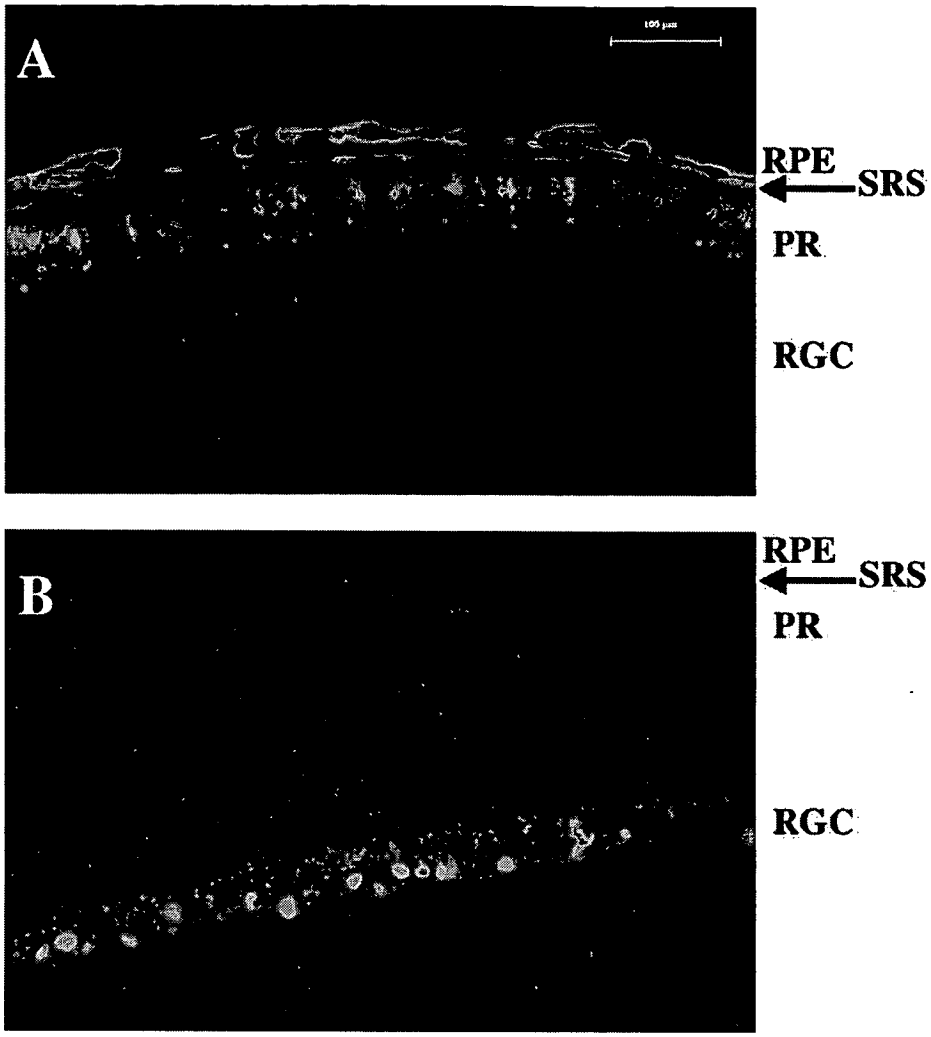

FIGURE 1. Dependence of retinal cell transduction on route of injection of AAV-CBA-GFP vector. Ocular sections were examined by fluorescence microscopy 6 weeks after subretinal (A) or intravitreous (B) injection of 3×10^9 particles of AAV-CBA-GFP. (A) After subretinal injection, prominent transduction occurred in RPE cells and photoreceptors (PR) bordering the subretinal space (SRS), but no detectable GFP appeared in retinal ganglion cells (RGC). (B) After intravitreous injection, prominent transduction occurred in retinal ganglion cells (and possibly displaced amacrine cells), but no detectable GFP appeared in photoreceptors or RPE.

MATERIALS AND METHODS

Production of Recombinant AAV Vectors Expressing PEDF

Cloning of human PEDF has been described.[13] Recombinant (r)AAV constructs were based on pTR-UF,[16] a viral vector plasmid in which an expression cassette, consisting of a cytomegalovirus (CMV) enhancer and a truncated chicken β-actin promoter-exon 1-intron 1 (together termed CBA), and a poliovirus internal ribosome entry sequence precede the PEDF cDNA, and a simian virus (SV)40 polyadenylation site follows it. The entire construct is flanked by inverted terminal repeat sequences from AAV-2. AAV-CBA-PEDF vector titers were 1.5×10^{12} or 2.0×10^{13} particles/mL. The control vector (UF12) was constructed identically, except that the coding region for green fluorescent protein (GFP) was substituted for the coding region of PEDF. It was used at 2.4×10^{12} or 4.0×10^{12} particles/mL. Contaminating helper adenovirus and wild-type AAV, assayed by serial dilution cytopathic effects or infectious centers, respectively, were lower than our detection limit of six orders of magnitude below recombinant AAV vector titers.

Mouse Model of Laser-Induced CNV

Adult C57BL/6 mice were given either an intravitreous injection of UF12 or AAV-CBA-PEDF by previously published techniques.[13] Intravitreous injections were performed with a Harvard pump microinjection apparatus and pulled glass micropipets. Each micropipet was calibrated to deliver 1 μL of vehicle containing the appropriate number of viral particles, on depression of a foot switch. The mice were anesthetized, pupils were dilated, and under a dissecting microscope, the sharpened tip of the micropipet was passed through the sclera, just behind the limbus into the vitreous cavity, and the foot switch was depressed. Subretinal injections were performed using a condensing lens system on the dissecting microscope, which allowed visualization of the retina during the injection. The pipet's tip was passed through the sclera posterior to the limbus and was positioned just above the

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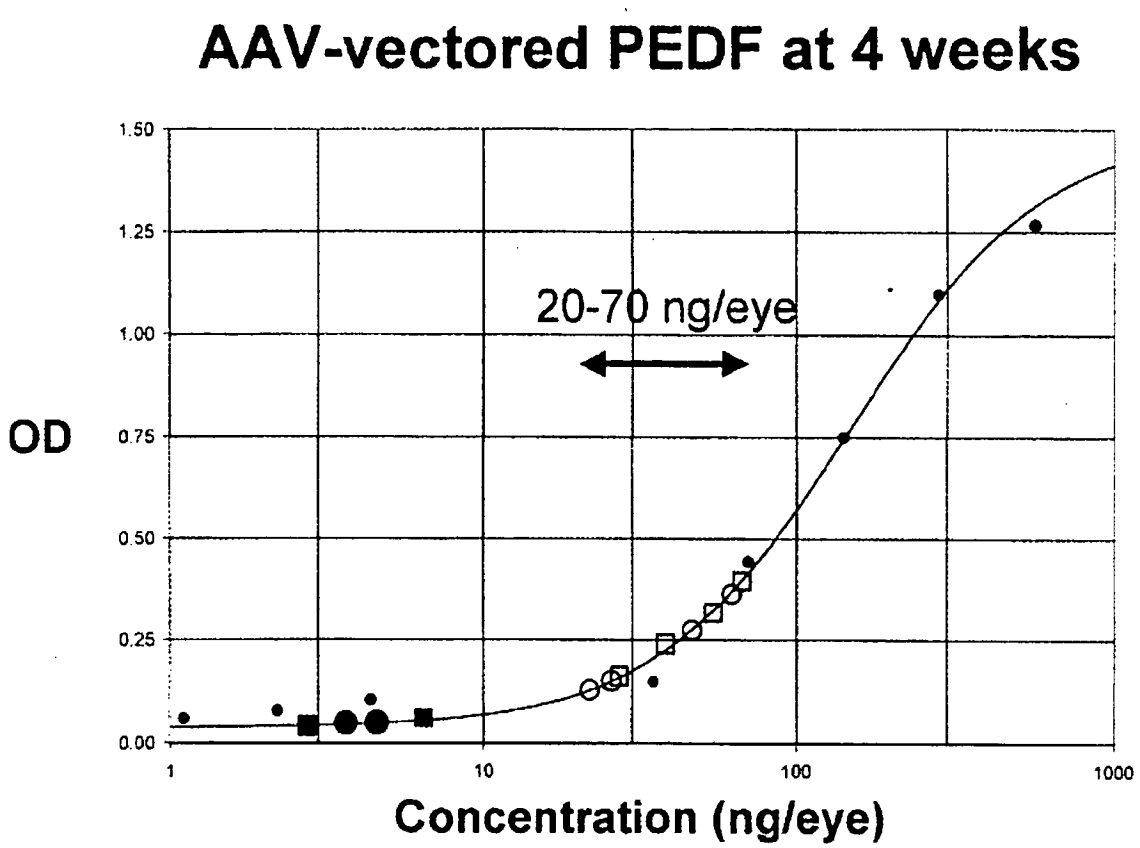

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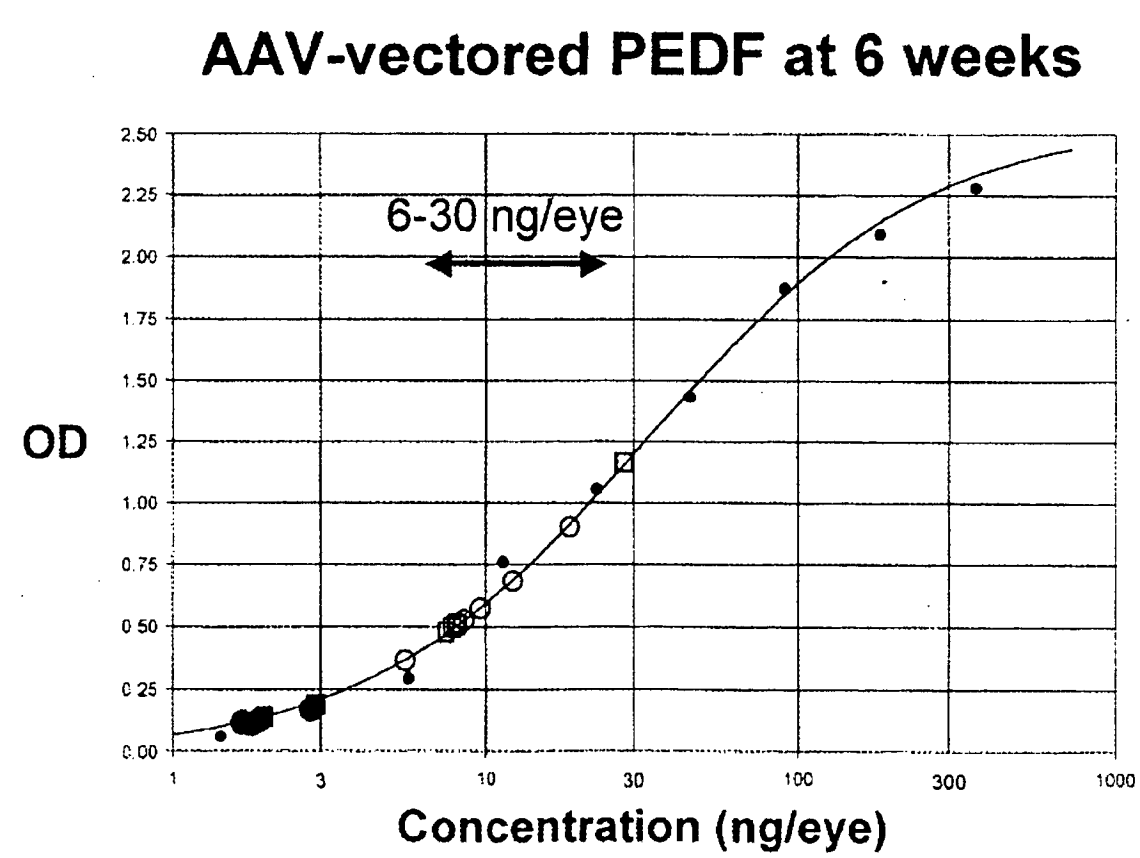

retina. Depression of the foot switch caused the jet of injection fluid to penetrate the retina. The blebs were quite uniform in size, and in each case, two of the laser burns were encompassed by the bleb, and one was outside the region of the bleb.

Two independent experiments were performed. In the first, mice were given intravitreous or subretinal injection of 1 μL containing 1.5×10^9 particles of AAV-CBA-PEDF or 4.0×10^9 particles of control vector, and 4 weeks after injection, the Bruch's membrane was ruptured with laser photocoagulation at three locations in each eye. Some mice were killed without treatment with laser photocoagulation, to measure ocular PEDF levels by ELISA. In the second experiment, mice were given intravitreous or subretinal injection of 1 μL containing 2.4×10^9 particles of control vector or 2.0×10^{10} particles of AAV-CBA-PEDF, and then 6 weeks after injection, the Bruch's membrane was ruptured by laser photocoagulation at three sites in each eye, as previously described.[17] Briefly, laser photocoagulation (532-nm wavelength, 100-μm spot size, 0.1-second duration, and 120-mW intensity) was delivered using the slit lamp delivery system and a handheld cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions two to three disc diameters from the optic nerve. Production of a vaporization bubble at the time of laser, which indicates rupture of the Bruch's membrane, is an important factor in obtaining CNV,[17] and therefore only burns in which a bubble was produced were included in the study.

Measurement of the Sizes of Laser-Induced CNV Lesions

Two weeks after laser treatment, the sizes of CNV lesions were measured in choroidal flatmounts.[18] Mice used for the flatmount technique were anesthetized and perfused with 1 mL phosphate-buffered saline (PBS) containing 50 mg/mL fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma, St. Louis, MO), as previously described.[19] The eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea and lens were removed, and the entire retina was carefully dissected from the eyecup. Radial cuts (four to seven; average, five) were made from the edge to the equator, and the eyecup was flatmounted in aqueous medium (Aquamount; BDH, Poole, UK) with the sclera facing down. Flatmounts were examined by fluorescence microscopy (Axioskop; Zeiss, Thornwood, NY), and images were digitized using a three-color charge-coupled (CCD) video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. The areas within each eye were averaged to obtain one experimental value, and mean values were calculated for each treatment group and compared by Student's unpaired *t*-test.

Some mice were killed 2 weeks after laser treatment, and eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN). Frozen serial sections (10 μm) were cut through the entire extent of each burn and histochemically stained with biotinylated *G. simplicifolia* lectin B4 (GSA; Vector Laboratories, Burlingame, CA), which selectively binds vascular cells. Slides were incubated in methanol-H_2O_2 for 10 minutes at 4°C, washed with 0.05 M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 minutes in 10% normal porcine serum. Slides were incubated 2 hours at room temperature with biotinylated GSA, and after rinsing with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 minutes at room temperature. After a 10-minute wash in 0.05 M TBS, slides were incubated with HistoMark Red (Kirkegaard & Perry, Cabin John, MD), to give a red reaction product that is distinguishable from melanin, and counterstained with Contrast Blue (Kirkegaard & Perry).

Fluorescence Microscopy after Intravitreous or Subretinal Injection of AAV-CBA-GFP

Adult male C57BL/6 mice were given either a subretinal or intravitreous injection of 3×10^9 particles of AAV-CBA-GFP. Six weeks after injection, mice were killed and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The eyes were removed, the cornea was punctured, and the eyes were immersed in the same fixative for 2 hours at 4°C. After removal of the cornea and lens, eyecups were cryoprotected in 30% sucrose in PBS for 6 to 12 hours and then frozen in OCT. Ten-micrometer frozen sections were mounted on gelatin-coated slides and used immediately or stored at −20°C. Slides were examined by fluorescence microscopy (Axioplan 2; Zeiss).

ELISA for PEDF

Mice were killed, and eyes were removed, quick frozen in 100 μL PBS (pH 7.4) with 0.05% phenylmethylsulfonyl fluoride, and homogenized manually on ice using a ground glass tissue homogenizer followed by three freeze–thaw cycles on liquid nitrogen and wet ice. The homogenate was centrifuged in a refrigerated desktop centrifuge to pellet the insoluble material, and the supernatant was loaded in sample wells for detection by ELISA. PEDF was detected by a sandwich ELISA procedure using a biotin-conjugated antibody and HRP-conjugated avidin for detection. Rabbit anti-PEDF was coated on 96-well, flat-bottomed microtiter plates (Immulon; Thermo Labsystems Oy, Helsinki, Finland) in 0.1 M $NaHCO_3$ overnight at 4°C. The wells were blocked with 10% fetal bovine serum in PBS (pH 7.4) for 2 hours at 37°C. PEDF protein standards and eye extract samples were loaded as 100-μL aliquots into wells, and the plate was kept overnight at 4°C. Detection consisted of a secondary mouse polyclonal anti-PEDF followed by a biotin-conjugated rat anti-mouse IgG (ICN Biomedicals, Costa Mesa, CA) and HRP-conjugated avidin (PharMingen, San Diego, CA). Each step of detection was conducted with plate agitation at room temperature for 1 to 2 hours, and the plate was washed five times between steps. A TMB peroxidase substrate system (Kirkegaard & Perry) was allowed to reach fully developed color, usually after 30 minutes, before the reaction was stopped with 1 M H_3PO_4. The plates were read in an automated microplate reader at 450 nm.

Results

Localization of Expression of the AAV-Vectored Transgene

Six weeks after subretinal injection of AAV-CBA-GFP, fluorescence microscopy showed prominent fluorescence from GFP in photoreceptors and RPE cells (Fig. 1A). In contrast, 6 weeks after intravitreous injection of AAV-CBA-GFP, there was prominent expression of GFP in ganglion cells (and possibly displaced amacrine cells), but no detectable expression in RPE cells or photoreceptors (Fig. 1B).

Expression of PEDF in Mice after Intravitreous or Subretinal Injection of AAV-CBA-PEDF

Mice given an intravitreous or subretinal injection of AAV-CBA-PEDF showed levels of human PEDF ranging from 20 to 70 ng/eye 4 weeks after the injection (Fig. 2A). In a second series

FIGURE 2. Intraocular levels of human PEDF 4 and 6 weeks after intraocular injection of control vector or AAV-CBA-PEDF. (**A**) C57BL/6 mice were given a subretinal (*squares*) or intravitreous (*circles*) injection of control vector (*filled*) or AAV-CBA-PEDF (*open*). Four (**A**) or 6 (**B**) weeks after injection, the mice were killed, and PEDF levels were measured in whole-eye homogenates by ELISA. The optical density (OD) of the standard concentrations (*small filled circles*) were plotted to generate the standard curve. The PEDF levels in eyes injected with control vector were below the limit of detection, and the levels in eyes injected with AAV-CBA-PEDF ranged from (**A**) 20 to 70 ng at 4 weeks (**B**) and from 6 to 30 ng at 6 weeks.

of mice, the range of PEDF was 6 to 30 ng/eye 6 weeks after intravitreous or subretinal injection of vector (Fig. 2B). All mice given intravitreous or subretinal injections of control vector had undetectable levels of PEDF. Given the variability from injection to injection, these ranges of PEDF are likely to be the same at 4 and 6 weeks after injection, and they are well above the background levels observed in control eyes. Subretinal and intravitreous injection of PEDF vector produced similar and overlapping levels of protein expression.

Effect of Intravitreal or Subretinal Injection of AAV-CBA-PEDF on CNV

Figure 3 illustrates representative flatmounts and cross-sections from the group of mice treated with laser 6 weeks after vector injection. Mice that did not receive an intraocular injection showed large areas of CNV at sites of rupture of Bruch's membrane (Figs. 3A, 3B). Control mice that received an intravitreous (Figs. 3C, 3D) or subretinal (Figs. 3E, 3F) injection of 2.4×10^9 particles of UF12 showed areas of CNV that were very similar to those in uninjected mice. Mice that received an intravitreous (Figs. 3G, 3H) or subretinal (Figs. 3I, 3J) injection of 2×10^{10} particles of AAV-CBA-PEDF showed visibly smaller areas of CNV than did uninjected mice or mice injected with UF12.

Measurement of the area of CNV by image analysis in each of the groups showed that there was no significant difference between the mean area in uninjected mice and mice given an intravitreous or subretinal injection of empty virus. Mice treated with laser 4 (Fig. 4A) or 6 weeks (Fig. 4B) after intravitreous or subretinal injection of AAV-CBA-PEDF showed significantly smaller mean areas of CNV than did mice injected with control vector.

Discussion

Current treatments for CNV are ineffective, because they are directed at ablating the new vessels, but do not address the underlying angiogenic stimuli. Using adenoviral vectors, we have recently demonstrated that two proteins that have previously been shown to inhibit tumor angiogenesis, endostatin and PEDF, also inhibit ocular neovascularization.[13,20] These studies also provide proof of concept for use of gene transfer to treat ocular neovascularization. In the present study, PEDF inhibited ocular neovascularization, independent of the vector used to express the PEDF.

Another recent study has demonstrated that systemic administration of recombinant PEDF protein inhibits retinal neovascularization in the murine model of oxygen-induced ischemic retinopathy.[21] In that study, the minimum effective dose of PEDF protein was approximately 5 μg, administered by daily intraperitoneal injections. Assuming that 5 μg was the steady state, whole-animal level and correcting for the fractional volume of the eye relative to the whole body (both conservative assumptions), the threshold level of PEDF necessary to inhibit retinal neovascularization is estimated at approximately 2 ng/eye. All PEDF vector-treated eyes exceeded this level, usually by one order of magnitude or more. Therefore, the ocular levels of PEDF after gene transfer in our study that resulted in inhibition of CNV are likely to be above the therapeutic level for inhibition of retinal neovascularization.

The demonstration that AAV-mediated intraocular expression of PEDF reduces CNV at sites of rupture of the Bruch's membrane is important with regard to practical aspects of treatment development. Patients with age-related macular degeneration (AMD) are at risk for the development of CNV for many years, and long-term treatment is needed. Prolonged intraocular transgene expression has been achieved with AAV

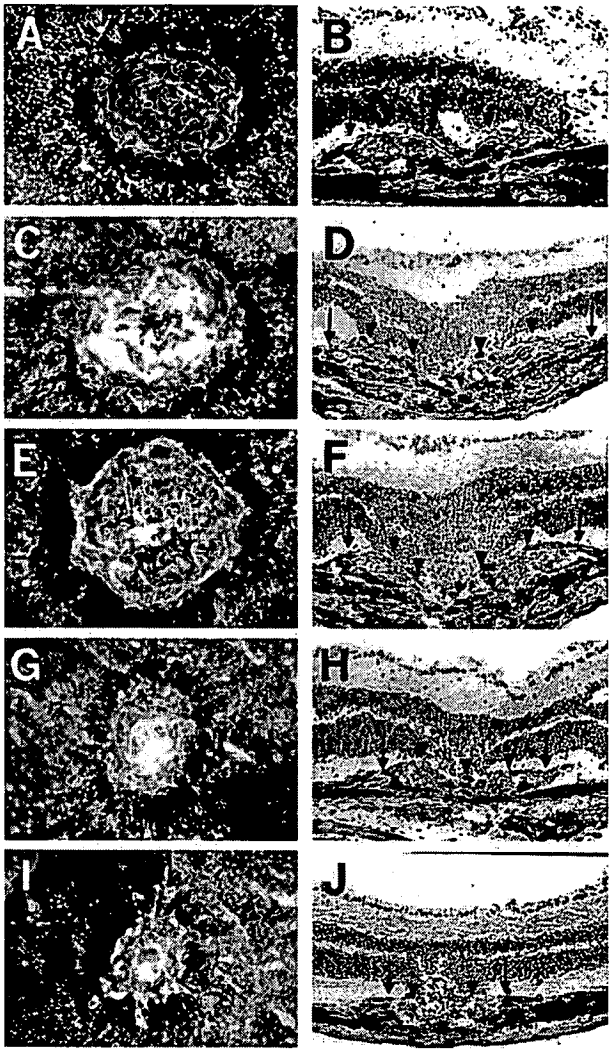

FIGURE 3. Smaller CNV lesions in eyes injected with AAV-CBA-PEDF compared with eyes injected with control vector. C57BL/6 mice were given an intravitreous or subretinal injection of control vector or AAV-CBA-PEDF. Six weeks after injection, the Bruch's membrane was ruptured with laser photocoagulation at three sites in each eye. Two weeks after rupture of the Bruch's membrane, the mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were prepared (A, C, E, G, I), or eyes were frozen and serial sections were stained with GSA lectin B4, which stains vascular cells, and counterstained with hematoxylin and eosin (B, D, F, H, J). The section showing the maximum diameter (*arrows*) for each CNV lesion is shown, and the thickness is indicated by the *arrowheads* along the surface. (A) Fluorescence microscopy shows a large CNV lesion at the rupture site of the Bruch's membrane in an eye that did not receive any injections. (B) A frozen section through the center of a CNV lesion in another uninjected eye shows a large maximum diameter (*arrows*). The lesion was thick, as shown by the *arrowheads* along its surface. Large CNV lesions were observed in eyes that received intravitreous (C) or subretinal (E) injection of control vector. A frozen section through the center of a CNV lesion in different eyes that received intravitreous (D) or subretinal (F) injection of control vector shows that the lesions had large maximum diameters (*arrows*). Small areas of CNV were observed in eyes that received intravitreous (G) or subretinal (I) injection of AAV-CBA-PEDF. A frozen section through the center of CNV lesions in different eyes that received intravitreous (H) or subretinal (J) injection of AAV-CBA-PEDF shows that the lesions had small maximum diameters (*arrows*).

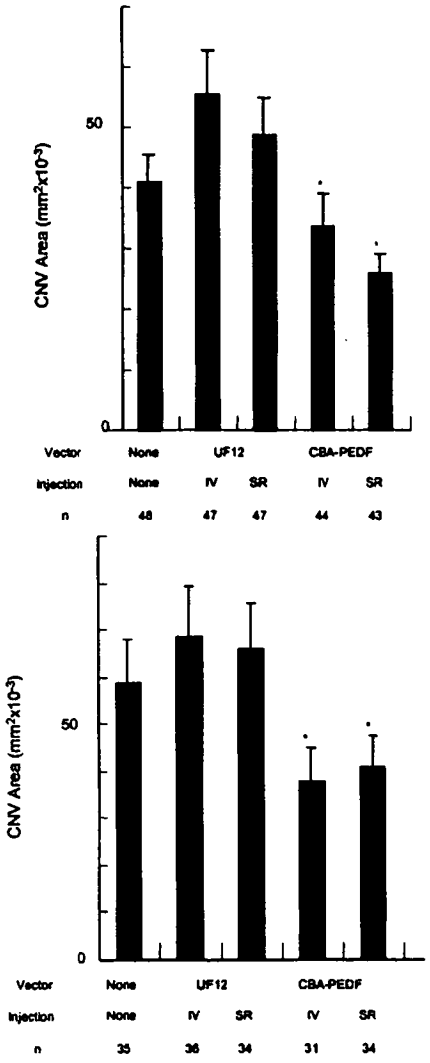

FIGURE 4. AAV-vectored PEDF inhibited CNV. Four weeks after intravitreous (IV) or subretinal (SR) injection of 4.0×10^9 particles of control vector (UF12) or 1.5×10^9 particles of AAV-CBA-PEDF (A) or six weeks after IV or SR injection of 2.4×10^9 particles of UF12 or 2.0×10^{10} particles of AAV-CBA-PEDF (B), C57BL/6 mice had laser-induced rupture of Bruch's membrane at three sites in each eye. Two weeks later, the mice were perfused with fluorescein-labeled dextran, choroidal flatmounts were prepared, and the area of CNV at each rupture site was measured by image analysis. $^*P < 0.05$ for difference from results of control vector administered by the same route, determined by unpaired *t*-test for samples with unequal variances.

vectors, and therefore they may provide the sustained intraocular production of antiangiogenic proteins that is likely to be needed to counter chronic production of angiogenic stimuli.

PEDF is a particularly appealing therapeutic candidate for patients with AMD. Although CNV is the major cause of severe visual loss in patients with AMD, most moderate loss of vision is due to death of photoreceptors and retinal pigmented epithelial (RPE) cells. PEDF was first identified as a component of conditioned medium of cultured fetal RPE cells that causes neurite outgrowth of Y79 retinoblastoma cells.[22,23] Several studies have suggested that PEDF has neuroprotective activity,[24-29] including protection of photoreceptors separated from the RPE from degeneration and loss of opsin immunoreactivity.[30] Therefore, long-term AAV-mediated expression of PEDF in the eyes of patients with early AMD may slow progression of the degeneration as well as reduce the likelihood of CNV.

Long duration of expression is not the only advantage of AAV vectors. Although use of adenoviral vectors is complicated by significant inflammation, there is no recognized AAV-mediated toxicity. Also, although AV vectors mediate higher levels of transgene expression after subretinal injections than after intravitreous injections,[13,31] there is comparable expression of PEDF after either intravitreous or subretinal injection of AAV-CBA-PEDF. This is probably because AAV-CBA vectors efficiently transduce ganglion cells, whereas adenoviral vectors do not. The attainment of comparable PEDF levels and efficacy after either intravitreous or subretinal injection of AAV-CBA-PEDF is important. From a clinical standpoint, intravitreous injections are easier and less invasive than subretinal injections, and whereas the former can be done in the clinic, the latter necessitates a procedure in the operating room.

The duration of AAV-mediated expression of proteins in the eye is not yet known. In rodents, expression appears to occur for the entire life of the animal (Flannery JG, Hauswirth WW, unpublished data). Although such long-term expression is an advantage from one viewpoint, it also raises potential concerns. If chronic expression of an antiangiogenic agent in the eye has some unsuspected deleterious effect, it may not be possible to halt the expression. Use of promoter systems that allow inducible expression could provide a safety net until the effects of long-term expression of PEDF in the eye are better understood. In any case, although further refinement of the system and more study are needed, the demonstration that AAV-mediated expression of PEDF in the eye inhibits CNV is an important step in the development of antiangiogenic gene therapy for patients with AMD.

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Adeno-associated virus type-2 expression of pigmented epithelium-derived factor or Kringles 1–3 of angiostatin reduce retinal neovascularization

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Neovascular diseases of the retina include age-related macular degeneration and diabetic retinopathy, and together they comprise the leading causes of adult-onset blindness in developed countries. Current surgical, pharmaceutical, and laser therapies for age-related macular degeneration (AMD) rarely result in improved vision, do not significantly prevent neovascularization (NV), and often result in at least some vision loss. To address this therapeutic gap, we determined the efficacy of recombinant adeno-associated viral (rAAV) serotype-2-mediated expression of pigment epithelium-derived factor (PEDF) or Kringle domains 1–3 of angiostatin (K1K3) in reducing aberrant vessel formation in a mouse model of ischemia-induced retinal NV. Both PEDF and K1K3 are potent inhibitors of NV when injected directly, hence expression of these therapeutic factors from rAAV may provide long-term protection from neovascular eye disease. rAAV vectors expressing the therapeutic gene were injected into one eye of postnatal day 0 (P0) newborn mouse pups. Retinal NV was induced in P7 mice by exposure to elevated oxygen for 5 days followed by room air for another five days. Retinal NV was quantified by the number of vascular-endothelial-cell nuclei above the inner-limiting membrane in P17 eyes. The number of such vascular endothelial cell nuclei in eyes treated with rAAV-PEDF or rAAV-K1K3 was significantly reduced (both $P < 0.0000002$) compared with control eyes. Ocular protein levels detected by ELISA correlate well with the reduction in NV and confirm that expression of antineovascular agents from rAAV vectors may be a therapeutically useful treatment of retinal or choroidal neovascular disease.

Control of the formation of new blood vessels in the retina is essential to the preservation of vision. Pathologic neovascularization (NV) of the retina is central to several debilitating ocular diseases including proliferative diabetic retinopathy (PDR), age-related macular degeneration (AMD), and retinopathy of prematurity (ROP). Diabetic retinopathy and AMD are the leading causes of blindness in developed countries. Regulation of vascularization in the mature retina involves a balance between endogenous positive growth factors, such as vascular endothelial growth factor (VEGF) (1, 2), and inhibitors of angiogenesis, such as pigment epithelium-derived factor (PEDF) (3). When this balance is upset, pathologic angiogenesis can occur, ultimately leading to a loss of vision.

Several clinical treatment options currently exist for patients presenting with retinal and choroidal NV (CNV). Surgical, laser, and photodynamic therapy (for AMD) techniques have been most commonly used to treat both PDR and AMD (4, 5). Panretinal laser photocoagulation has been used with relative success for a number of years (6, 7). Unfortunately, laser photocoagulation can also damage healthy cells adjacent to or underlying the treated area leading to a significant loss of peripheral and night vision. Laser photocoagulation for CNV secondary to AMD often leads to an immediate significant reduction in visual acuity (8). A more recent development is the use of photodynamic therapy to treat CNV that reduces collateral damage during laser therapy (9, 10–12). Surgical treatments include vitrectomy for the removal of CNV, foveal translocation, and the removal or displacement of subretinal blood for AMD patients and the removal of vitreous hemorrhage and scarring secondary to fibrovascular proliferation in PDR patients (13–15). These surgical interventions carry intrinsic risks to the patient and can create further complications (4, 5). All current treatment options provide solutions that result in some loss of vision and may have only temporary effects. Recurrence of symptoms is common and may ultimately result in loss of vision. Clearly, the need exists for therapies that require minimal surgical manipulation, preserve existing vision, and provide long-term amelioration for any form of NV.

Two of the most potent general inhibitors of neovascularization are Kringle domains 1 through 3 of angiostatin (K1K3) and PEDF. K1K3 is a proteolytic fragment of plasminogen that retains potent angiostatic properties. It is an endogenous regulator of vasculogenesis and, as a naturally occurring peptide, it is not likely to stimulate an immunogenic response (16, 17). Neither plasminogen nor plasmin inhibits endothelial cell proliferation, nor does angiostatin affect coagulation. Although angiostatin is known to inhibit tumor growth *in vivo* by increasing apoptosis and inhibiting tumor-associated angiogenesis, its precise mechanism of action is unclear. Apoptosis *in vitro* is induced in endothelial cells by multiple forms of angiostatin (18), and cells have been shown to be arrested at the G_2/M transition interface (19). Administration of angiostatin to tumor-bearing mice has not resulted in detectable systemic cytotoxicity; only angiogenic proliferation appears to be inhibited (20–22). Angiostatin, therefore, appears to be an effective and nontoxic inhibitor of NV that is worth evaluating in models of ischemic retinopathy. A recent study has indicated that Kringle 5 of angiostatin may induce PEDF and inhibit VEGF, both in cell culture and in a rat model of ischemic retinopathy (23).

PEDF, purified from human retinal pigment epithelial cultures as a factor that induces neuronal differentiation of cultured retinoblastoma cells (24, 25), has been recently shown to regulate normal angiogenesis in the eye (3). PEDF is found both intracellularly and extracellularly in the fetus and early adult eye, but is lost at the onset of senescence (26, 27). It is down-regulated by hypoxia and induced in the retina as a result of hyperoxia, is a very potent inhibitor of corneal NV, and prevents endothelial cell migration toward a wide variety of angiogenic inducers (3). PEDF, therefore,

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Abbreviations: NV, neovascularization; CNV, choroidal NV; AMD, age-related macular degeneration; rAAV, recombinant adeno-associated virus; CBA, chicken β-actin; PEDF, pigment epithelium-derived factor; K1K3, Kringle domains 1–3 of angiostatin; PDR, proliferative diabetic retinopathy; ROP, retinopathy of prematurity; P*n*, postnatal day *n*; ILM, inner limiting membrane.

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appears to be a major angiogenic regulator of the retinal vasculature and is an excellent candidate gene for therapy against ocular NV. As an intraocularly injected protein, PEDF delays the loss of photoreceptors in the rd mouse (28), implying that it may also possess neurotrophic activity in the retina and that the extracellular protein can effectively disperse throughout the retina. We hypothesize here that when expressed in a secretable form as a virally vectored gene, either PEDF or K1K3 may be relatively independent of the retinal cell type supporting expression and may be effective in limiting retinal NV.

To determine whether PEDF, K1K3, or both are potentially useful for therapeutic control of retinal NV, we examined the effect of expression of the angiostatic factors PEDF and K1K3 in a mouse model of ischemic retinopathy. We chose recombinant adeno-associated virus (rAAV) serotype-2 to deliver the therapeutic genes because issues regarding attainment of high vector titers have been resolved (29) and rAAV preparations free of contaminating replication-competent rAAV are now routine (29, 30). rAAV-mediated gene delivery results in long-term expression in a wide variety of tissues, including various cell types in the retina (31) and optic nerve (32). Finally, rAAV vectors have shown very little *in vivo* toxicity in a variety of tissues (33).

Materials and Methods

Animals. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were obtained from the Jackson Laboratory. Breeding pairs of mice were housed in the University of Florida Health Science Center Animal Resources facilities. Females were examined daily for signs of pregnancy and isolated in individual cages for confirmation. Timed-pregnant dams were also occasionally obtained from the same vendor. Animals were euthanized by an overdose of ketamine/xylazine mixture given s.c. (10 μg/g body weight ketamine·HCl, 2 μg/g body weight xylazine in an appropriate volume of 0.9% NaCl).

Vector Design, Packaging, and Delivery. The rAAV-vector cassette consists of a selected promoter upstream of a simian virus 40 early splice donor/splice-acceptor site, the expressed gene, and a simian virus 40 polyadenylation sequence. PEDF cDNA was a gift of D. Zack (Johns Hopkins University, Baltimore) and K1K3 cDNA was a gift of P. Meneses (Weill Medical College of Cornell University, New York). K1K3 has an IgK leader-peptide secretory sequence upstream of the expressed gene. At its carboxyl terminus, K1K3 also has a myc epitope for ELISA detection. The entire expression cassette containing either cDNA is flanked by adeno-associated virus2 terminal repeats required for viral packaging. Viral vectors were packaged and purified as described (29, 30). Briefly, the vector cassette is transfected into human 293 cells along with a helper plasmid containing adeno-associated virus and adenovirus helper functions. The cells are harvested in PBS with EDTA, pelleted, and resuspended in a low-salt buffer, and lysed by freeze–thaw. The virus is purified on an iodixinol gradient followed by heparin sulfate affinity column chromatography. rAAV vector is eluted from the heparin-agarose matrix with 1 M NaCl and concentrated. Initial viral titer is calculated by quantitative competitive PCR and a final titer is determined by infectious center assay.

To determine the optimum promoter to drive expression of the therapeutic gene, we designed five promoter constructs. Each promoter regulates expression in a subset of cells in the retina. Chicken β-actin (CBA) is a ubiquitous strong promoter composed of a cytomegalovirus (CMV) immediate-early enhancer (381 bp) and a CBA promoter-exon1-intron1 element (1,352 bp). *cis*-Retinaldehyde-binding protein (CRALBP) promoter is a retinal-pigment-epithelium (RPE)-specific promoter (2,265 bp) when administered subretinally in a rAAV vector (A. Timmers and W.W.H., unpublished data). Mouse rod opsin (MOPS) is a photoreceptor-specific promoter (31) (472 bp); platelet-derived growth factor (PDGF) is an endothelial promoter (1,600 bp) that regulates expression in retinal ganglion cells when delivered intravitreally in rAAV vectors (A. Timmers and W.W.H., unpublished data); and the CMV promoter (620 bp), like CBA, is also relatively ubiquitous in the retina but expresses at a lower levels than CBA (34). We inserted each of these promoters upstream of the therapeutic genes, PEDF or K1K3. For the purpose of determining *in vivo* expression levels, each vector was injected into the subretinal or intravitreal space of adult mice. Approximately 10^{10} particles (2×10^8 infectious units) in a volume of 1 μl of therapeutic vector was injected into the right eye either subretinally or intravitreally. The contralateral eye was injected with the same volume of PBS. Mouse pups were injected intraocularly with 0.5 μl on postnatal day (P)0 with one of the experimental vectors in the right eye and either no injection or PBS in the contralateral eye.

ELISA. Eyes from age-matched animals were enucleated and quickly frozen in 100 μl of PBS, pH 7.4/0.05% PMSF, and manually homogenized on ice by using a ground-glass tissue homogenizer, followed by three freeze–thaw cycles on liquid nitrogen and wet ice. The homogenate was centrifuged in a refrigerated desktop centrifuge at 5,000 $\times$ *g* for 2 min to pellet the insoluble material. The resulting whole-eye extract was loaded into sample wells for detection by ELISA. We determined the ocular levels of PEDF protein by an indirect sandwich ELISA procedure by using a biotin-conjugated final antibody and horseradish peroxidase (HRP)-conjugated avidin for detection. Rabbit anti-PEDF (gift from P. Campochiaro, Johns Hopkins University, Baltimore) was coated on 96-well Immulon flat-bottom microtiter plate (Dynex Technologies, Middlesex, U.K.) in 0.1 M $NaHCO_3$ overnight at 4°C. The wells were blocked with 10% FBS in PBS, pH 7.4, for 2 h at 37°C. We then loaded PEDF protein standards and eye-extract samples as 100-μl aliquots into the wells and incubated the plate overnight at 4°C. Detection consisted of a secondary mouse polyclonal anti-PEDF (gift of P. Hargrave, University of Florida, Gainesville) followed by a biotin-conjugated rat anti-mouse IgG (ICN) and HRP-conjugated avidin (PharMingen). Each step of the detection was conducted with plate agitation at room temperature for 1–2 h and the plate was washed five times between steps. TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate system (Kirkegaard & Perry Laboratories) was pipetted into all wells and allowed to reach fully developed color, usually for 30 min, before stopping the reaction with 1 M H_3PO_4. The plates were read by absorbance at 450 nm in an automated microplate reader. A similar method was used to determine K1K3 levels. Rat anti-K1K3 (Enzyme Research Laboratories, South Bend, IN) was coated onto plates, and K1K3 samples were loaded as described. We then used a biotinylated secondary antibody to the myc epitope (Invitrogen) on the expressed K1K3 to detect bound protein. HRP-conjugated avidin followed by TMB peroxidase substrate completed the detection step as described above.

Hyperoxia Treatment. Mouse pups, with their nursing dam, were placed in a chamber at 73% oxygen at P7 and maintained in this environment for 5 days until P12. At this time the pups and nursing dam were returned to normal room air and maintained for another 5 days. At P17, the pups were euthanized as described above and their eyes were enucleated and fixed for embedding and sectioning. Representative pups from each group were anesthetized and perfused through the left ventricle with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) containing 5 mg/ml FITC-dextran for visualizing the retinal vasculature (see below).

Table 1. Ocular K1K3 or PEDF levels for various viral vector constructs

Vector construct	PEDF, ng per retina	K1K3, ng per retina
CBA	20–70	6–60
CRALBP	3–4	<1
MOPS	1–2	<1
PDGF	2–3	3–4
CMV	<1	<1
CBA (neonatal)	1–8	2–3

Expression of K1K3 or PEDF was determined by ELISA from whole eye homogenates. rAAV vectors with the CBA promoter produced the highest levels of K1K3 or PEDF expression from either intravitreal or subretinal inoculations. There was no significant difference between intravitreal and subretinal injection for either K1K3 or PEDF. Neonatal expression levels measured between P2 and P17 for the CBA promoter for both K1K3 and PEDF are lower than the adult but still above the estimated therapeutic threshold (see *Discussion*). MOPS, mouse rod opsin; CRALBP, *cis*-Retinaldehyde-binding protein; CMV, cytomegalovirus.

Quantitative and Qualitative Assessment of Retinal NV. Both eyes of each P17 pup were enucleated and fixed for paraffin embedding and serially sectioned at 5 μm thickness as described by Smith *et al.* (35). Representative sections (every 30th section) through the full eyecup were stained with hematoxylin and eosin to visualize cell nuclei. Trained investigators masked to the identity of each section counted all endothelial cell nuclei above the internal limiting membrane in all representative sections through each eye. Vascular cell nuclei were considered to be associated with NV if they were on the vitreous side of the internal limiting membrane. Data were analyzed by a paired *t* test with vector-treated and contralateral uninjected eyes serving as determinants. For qualitative assessment of retinal NV both eyes of each perfused P17 pup were enucleated and the retina was dissected and flat-mounted as described by D'Amato *et al.* (36). Flatmounted retinas were photographed by fluorescence microscopy using a Zeiss Axioplan2 microscope, Zeiss Plan-Fluar 10× lens, Sony DXC-970MD camera with tile field imaging, and MCID software (Imaging Research, St. Catherine's, ON, Canada). At least three eyes from each treatment group were examined in this way.

Results

Optimizing Ocular Expression from the Vector Constructs. To determine the optimal promoter construct for use in antiangiogenic experimental therapies, we initially assayed the amount of ocular protein in adult mice expressed from each of five vector constructs containing different promoters. To a first approximation the level of PEDF or K1K3 protein expression in the eye should correlate with how well that vector performed in reducing aberrant NV. We chose a set of promoters that restrict the expression of genes to different specific subsets of retinal cells. Each vector-promoter construct was tested by either intravitreal or subretinal vector inoculation. We measured ocular protein expression levels by ELISA 6 weeks after vector injection. Only the nature of the promoter regulating the PEDF or K1K3 or the site of intraocular injection was changed. ELISA has advantages over other methods to examine the protein expression in the eye. PCR-based methods of determining promoter efficiency reflect only the amount of mRNA made and may not accurately reflect mature protein levels. ELISA provides a direct quantification that is more precise than Western blotting. The results are summarized in Table 1 and indicate that for both PEDF and K1K3 the CBA-hybrid promoter produces the most consistently robust ELISA measurements of protein expression. Interestingly, subretinal or intravitreal routes of vector delivery generated approximately equivalent levels of ocular protein. For purposes of maximizing protein expression in the retina, we therefore conclude that rAAV-CBA-PEDF and rAAV-CBA-K1K3 vectors are best suited for therapeutic evaluation in a retinal NV setting.

Vector Behavior in the ROP Mouse Model. To functionally test whether PEDF or K1K3 vectors reduced NV *in vivo*, we chose to examine their performance in a mouse model of ischemic retinopathy. Retinal NV is induced in a modification of a described protocol (35, 36). However, before analysis of any therapeutic effects, we needed to know what levels of each agent we could produce in the eyes of P1 to P17 neonatal mice because our initial survey of intraocular levels of passenger-gene expression was in adult mice with a mature retina. P1 neonatal mice were injected intraocularly with rAAV-CBA-PEDF or rAAV-CBA-K1K3 as described above. Levels of PEDF and K1K3 expression were followed by ELISA after injection. Expression of PEDF was detectable as early as P2 and persisted at levels well above contralateral uninjected eyes to the last time point at P17. The PEDF levels measured in the neonatal mice (1–8 ng per eye) were not as high as those measured in adult mouse eyes (20–70 ng per eye), however; they were 4- to 16-fold above levels in the untreated contralateral eyes. K1K3 levels measured in injected eyes of neonatal mice were 2–3 ng per eye. Quantitative differences between PEDF and K1K3 levels achievable in adults vs. neonates are likely related to size, developmental differences, and the time after injection.

Assessment of Antineovascular Effects. To qualitatively assess retinal NV after vector treatment we viewed the retinal vasculature in flat-mounted FITC-dextran-perfused whole retinas. Whole mounts were imaged using tile-field-mapping fluorescent microscopy, which allows the whole retina to be examined at high resolution. Such visual assessment of full retinal vascular beds provides a useful initial comparison between treatment groups. The patterns of NV observed in the FITC-dextran-perfused retinas were consistent with what has been reported in this model (refs. 35–37; Fig. 1). Retinas of neonatal mice exposed to hyperoxia exhibit increased tortuosity of radial vessels accompanied by increased perfusion of peripheral vessels and absence or reduction of perfusion in central vessels (Fig. 1*a*). For comparison, a whole mount from the normoxic age-matched animal shows the normal pattern of retinal vasculature (Fig. 1*b*). The uninjected, hyperoxia-exposed retina in Fig. 1*a* can be directly contrasted with the contralateral PEDF- (Fig. 1*c*) or K1K3- (Fig. 1*d*) vector-treated retinas. PEDF- or K1K3-vector-treated retinas showed a decrease in the number of neovascular tufts with a relatively uniform perfusion pattern over the full retina, and the overall vasculature pattern appeared much more like that in the normal animal (Fig. 1*b*). However, these sorts of images do not lend themselves readily to a quantitative analysis of NV, and are subject to several preparation artifacts (38). Therefore, an independent and more quantifiable additional analysis of the treatment groups was performed.

To gain better insight into the efficacy of treatment with rAAV-CBA-PEDF or rAAV-CBA-K1K3, direct enumeration of endothelial cells in the retinal vasculature was assessed as described (35). We enucleated and fixed both vector-treated and control eyes from P17 pups for paraffin-embedded sectioning. Representative sections spanning the entire retina provide a reliable method for quantitatively assessing the total level of retinal NV in each eye. Individuals masked as to the identity of the treatment groups quantified NV by enumerating all endothelial cell nuclei found in the vitreous space above the inner limiting membrane (ILM). Comparisons were made between one eye of each animal injected with therapeutic vector and the contralateral uninjected eye serving as an internal control (Fig. 2). In tissue sections, uninjected control eyes typically showed abundant longitudinal and transverse aberrant microvessels in

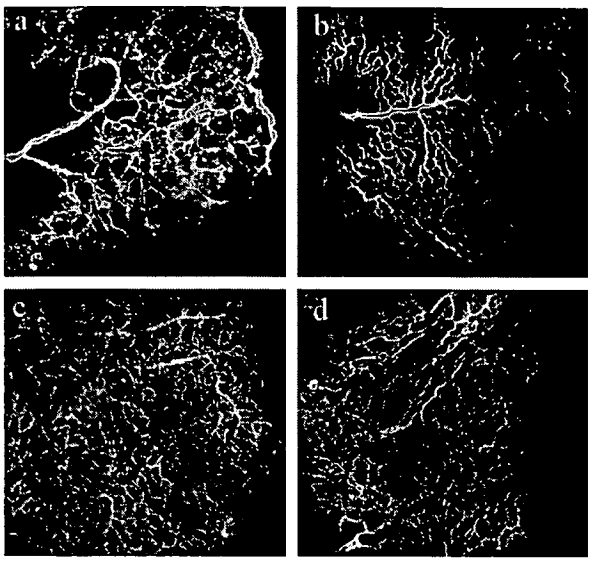

Fig. 1. Qualitative determination of neovascularization from composite tile-field-mapped ×10 photomicrographs of whole-mounts of retinas from 17-day mouse pups. The pups were perfused with FITC-dextran in 4% formaldehyde to visualize the retinal vasculature. Each panel shows one quadrant of the retinal whole-mount oriented with the central retina on the left and the peripheral on the right. (a) An untreated control pup exposed to 5 days of hyperoxia followed by 5 days at room oxygen exhibited the expected abnormal retinal vasculature. Note the increased peripheral perfusion, the dilated, tortuous radial vessels, and the largely avascular central retina. (*b*) A normoxic age-matched animal shows the normal pattern of retinal vasculature. ROP animals injected with either PEDF vector (*c*) or K1K3 vector (*d*) exhibited a vasculature much closer to that seen in a normal age-matched animal, with uniform perfusion over the retina and fewer areas of apparent vascular leakage.

the vitreous space above the ILM (Fig. 2*c*). Eyes injected with K1K3 or PEDF vector had a dramatically reduced number of aberrant vessels (Fig. 2 *a* and *b*, respectively). Vehicle-injected eyes and eyes injected with rAAV-CBA-GFP did not significantly differ from uninjected eyes in this assay (data not shown). Control eyes injected with rAAV-CBA-GFP demonstrate that the therapeutic effect is because of the expressed passenger genes and not because of the vector itself. Treatment with either rAAV-CBA-PEDF or rAAV-CBA-K1K3 significantly reduced the neovascular response (both $P < 0.0000002$) when compared with the paired uninjected control eye (Fig. 3). Average endothelial cell counts in PEDF-treated eyes were reduced by 74% compared with paired controls and 78% compared with paired controls for K1K3-treated eyes.

Discussion

Effective, long-lasting treatment of retinal neovascular disorders, including diabetic retinopathy, remains one of the greatest challenges in ophthalmology today. The number of individuals suffering from diabetes has increased worldwide in recent years and is projected to continue to rise (39). PDR is a common complication in diabetic patients. PDR shares a pathophysiology with our model of ischemic retinopathy because the initial ischemic insult and the subsequent pathologic outgrowth of new vessels from the retinal vasculature occur in both PDR and ROP and ultimately lead to blindness in either disease. Retinal NV can also occur secondarily to CNV in AMD (40, 41).

Although the minimum effective intraocular dose of PEDF or K1K3 for controlling ischemic retinopathy is unknown, previous studies following the administration of PEDF protein in the

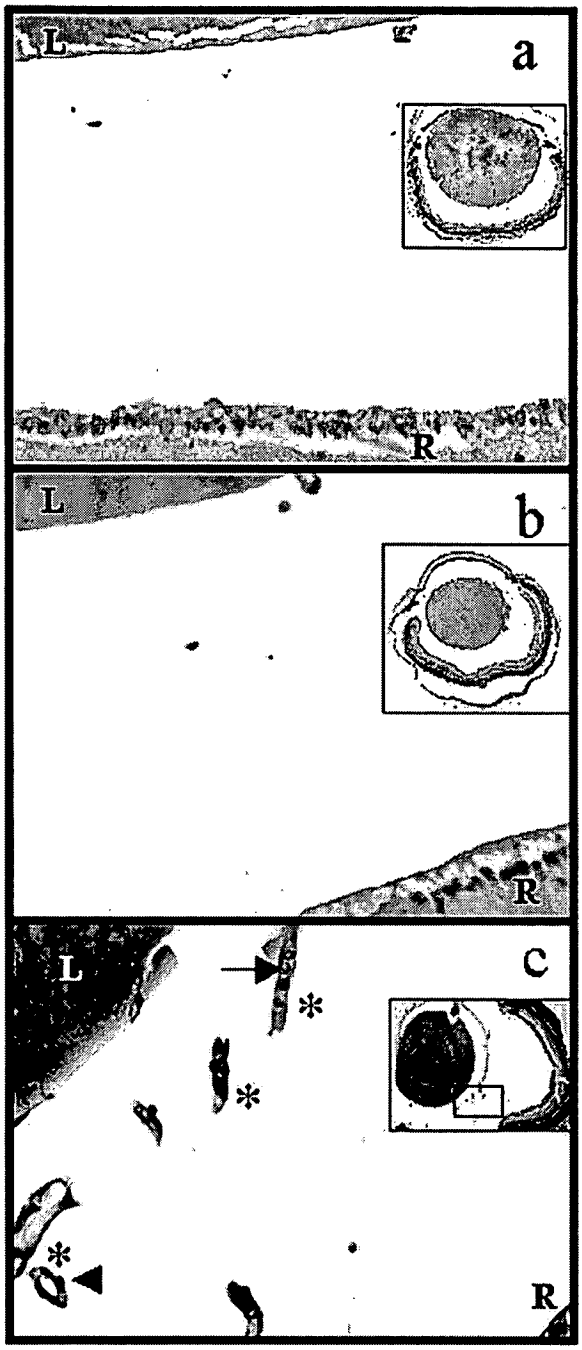

Fig. 2. Photomicrographs showing a portion of transverse sections of whole eyes from hyperoxia-treated mouse pups. Tile-field-mapped photomicrographs from representative whole eyes are inset in each panel. The arrowhead indicates a representative vascular endothelial cell nucleus and the arrow indicates a red blood cell within a longitudinal section of an abnormal microvessel (indicated by asterisks) that has penetrated the ILM. The vitreal space into which new blood vessels have penetrated is defined anteriorly by the lens (L) and posteriorly by the retina (R). Assessment of neovascularization requires quantitation of endothelial cell nuclei occurring on the vitreous side of the ILM (see Fig. 3). Eyes injected with rAAV-CBA-K1K3 had fewer endothelial cell nuclei in representative sections (*a*) than uninjected control eyes (*c*). Similarly, rAAV-CBA-PEDF treatment resulted in a reduced number of endothelial cell nuclei (*b*) compared with uninjected control eyes (*c*).

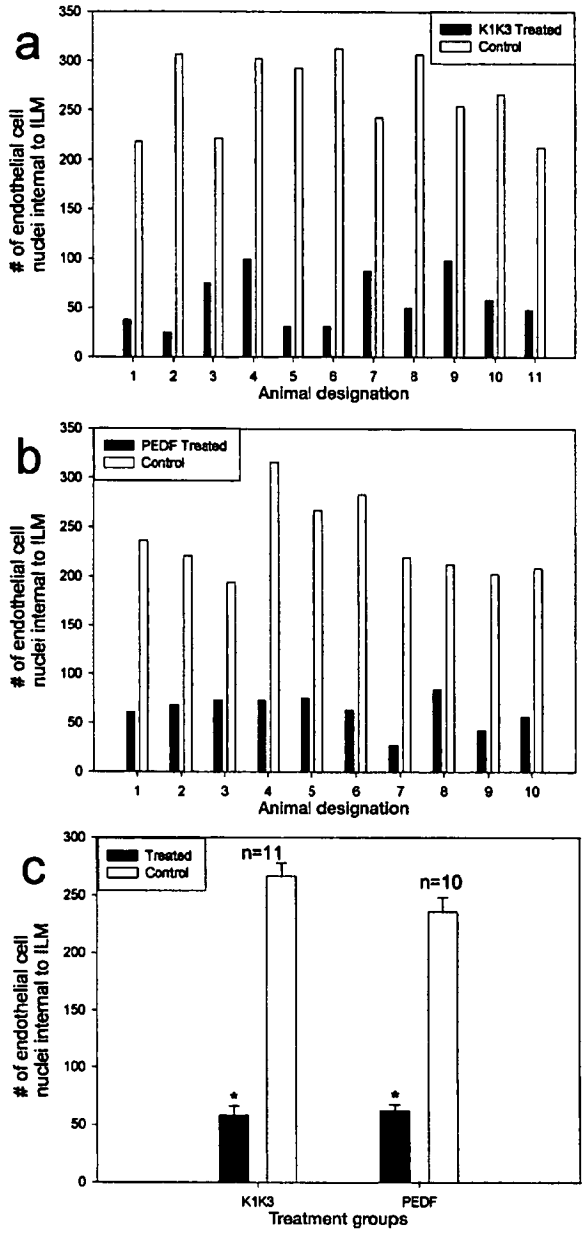

Fig. 3. Enumeration of endothelial cell nuclei above the ILM in sections of whole eyes from hyperoxia-treated P17 mouse pups. These quantitative results agree with the qualitative assessment (see Fig. 1), that vector-mediated expression of PEDF (*a*) or K1K3 (*b*) reduces the level of oxygen-induced NV. (*c*) Statistical analysis of the paired eyes shows a significant difference between control and treated eyes for both PEDF and K1K3 ($P < 0.0000002$).

same model we employ here showed the therapeutic threshold to be ≈5–11 μg/day (42) when administered systemically. If we make the simplest and most conservative assumptions, that PEDF is stable and partitioned into the eye from the systemic vasculature based simply on the volume of the eye vs. the volume of the whole animal, the concentration of ocular PEDF needed to inhibit retinal NV is estimated to be 1–2 ng per eye. This conservative calculation does not consider any pharmacokinetic stability parameters or the existence of the blood–retinal barrier, both of which would tend to reduce further the amount of PEDF reaching the eye from the circulation. It is therefore possible that the true intraocular therapeutic threshold for PEDF is much lower than this rough estimate. We demonstrated here that rAAV-vector-expressed PEDF produces intraocular concentrations of 20–70 ng in the adult mouse and 1–8 ng in the neonatal mouse. This is at or considerably higher than our estimated therapeutic threshold. Similar calculations based on another study in which one dose of K1K3 angiostatin administered systemically was able to effectively reduce retinal NV in the ROP mouse (43) yielded an estimated maximum intraocular therapeutic threshold of 1.6 ng per eye for K1K3. Our rAAV-vector expressing K1K3 angiostatin produces levels of 6–60 ng per eye in the adult or 2–3 ng per eye in the neonate, again, above our estimated maximum threshold. Therefore, it appears that we can achieve and maintain intraocular levels of either PEDF or K1K3 angiostatin in neonatal and adult mice sufficient to expect significant reduction of retinal NV.

Administration of rAAV vectors expressing cDNAs for antiangiogenic proteins can therefore be as effective as the administration of the corresponding proteins systemically, but without the requirement for repeated injections. In addition, use of rAAV vectors offers several potentially important advantages over the systemic administration of antiangiogenics. Local production and secretion of PEDF or K1K3 through vector gene delivery is likely to restrict any antineovascular activity to an area specific to the pathological angiogenesis in the subject. Proteins produced in the posterior ocular compartment are not likely to interfere with the normal angiogenic processes necessary for wound healing or tissue repair elsewhere in the body, and perhaps not even in the anterior segment of the same eye, although this remains to be tested. Further, rAAV vectors have demonstrated long-term, sustained high-level expression in the retina (32), and we observe nanogram levels in rat eyes for at least 21 months (B.J.R. and W.W.H., unpublished data), indicating that a single injection of rAAV bearing a therapeutic gene could provide durable inhibition of NV. This could obviate the need for repeated injections of antiangiogenic compounds systemically or intraocularly.

For the purposes of maximizing protein expression in the retina, we found that rAAV-CBA-PEDF and rAAV-CBA-K1K3 vectors produced the highest levels of ocular protein. The lower levels of expression seen for vectors with MOPS, CRALBP, and PDGF promoters may be due to the different cell specificity of these promoters compared with CBA, or, in the case of CMV, to a generally lower ability to support transcription in the same set of retinal cell types. When subretinally injected, CBA supports expression well in both retinal-pigment-epithelium (RPE) cells and photoreceptors (44). In contrast, the MOPS promoter is largely rod-photoreceptor-specific (31) and yields much lower intraocular expression, thus suggesting that the key source of secreted PEDF or K1K3 from vectors injected subretinally is the RPE cell. When injected into the vitreous, CBA-containing rAAV vectors express very well predominantly in retinal ganglion cells (45). Since there was no significant difference in the antineovascular effectiveness of subretinally or intravitreally administered PEDF or K1K3 vectors with the CBA promoter, it appears that the potentially less traumatic intravitreal route of vector delivery may be favored.

For optimizing the safety of ocular gene therapy for NV diseases it may be important to limit the expression of a therapeutic protein even more specifically, either to just a single retinal cell type or to a more defined topologic area. By altering the promoter used to drive PEDF or K1K3 expression it may be possible to fine-tune therapeutic gene expression to a pharmacologically significant but highly localized cellular pattern. The CBA promoter drives expression in multiple cell types, whereas a more specific promoter could be used to target expression

selectively. Alternatively, delivering the vector specifically to the intravitreal or subretinal space in a larger human eye may also define the localization of expression. Advanced stages of AMD are characterized by a NV of the chorocapillaris within or adjacent to the macula where treatment might be most effective if the therapeutic vector is administered subretinally near potentially active CNV regions. This type of subretinal administration effectively limits the lateral spread of vector-mediated expression (44), whereas vitreal administration may allow less constrained vector diffusion to a wider and less controlled retinal area. Full testing of these ideas will require development of an animal model with a retinal area closer in size to that in humans than that of the mouse.

In summary, we have demonstrated that rAAV vectors incorporating a CBA promoter are capable of producing sustained therapeutic levels of PEDF and K1K3 in the mouse eye. Intraocular injection of rAAV-CBA-PEDF or rAAV-CBA-K1K3 significantly reduced the level of retinal NV in a mouse model of ischemic retinopathy. Other studies have recently demonstrated effective gene therapy approaches for controlling ocular angiogenesis. Expression of PEDF from either rAAV (45) or adenovirus (46) vectors was effective in reducing CNV in rodent models. Adenoviral vectors expressing endostatin (47) or plasminogen-activator inhibitor-1 (48) were also effective in inhibiting retinal NV. Furthered by our present report, the generality of efficient and well-targeted gene-based approaches for treating neovascular diseases of the eye coupled with the potential of rAAV vectors for persistently delivering antiangiogenic proteins to the retina is becoming apparent.

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